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13. ABSTRACT (Maximum 200 words) Crimean-Congo hemorrhagic fever (CCHF) virus was transmitted from infected adult Hyalomma ticks to uninfected larval and nymphal Hyalomma ticks while cofeeding on a guinea pig host that did not have a detectable viremia. When tested after feeding with infected adults, three (0.8%) of 370 H. truncatum larvae contained detectable CCHF virus (mean virus titer $10^{1.6}$ plaque-forming units (PFU/tick). The virus was transmitted transstadially from infected larvae and was detected in 15 (1.2%) of 1,253 nymphs and 12 (0.1%) of 2,049 adults. Virus was recovered from 18 (1.9%) of 931 H. impeltatum nymphs, which originated from larvae that cofed with infected adults, CCHF virus was detected in 21 (4.3%) of 449 (mean virus titer $10^{1.7}$ PFU/tick) fed nymphs, but none of 886 adults tested after molt. Results of this study indicate that a small proportion of either larvae or nymphs may acquire CCHF infection while cofeeding on a host without a detectable viremia.

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TRANSMISSION OF CRIMEAN-CONGO HEMORRHAGIC FEVER VIRUS IN TWO SPECIES OF *HYALOMMA* TICKS FROM INFECTED ADULTS TO COFEEDING IMMATURE FORMS

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Abstract. Crimean-Congo hemorrhagic fever (CCHF) virus was transmitted from infected adult *Hyalomma* ticks to uninfected larval and nymphal *Hyalomma* ticks while cofeeding on a guinea pig host that did not have a detectable viremia. When tested after feeding with infected adults, three (0.8%) of 370 *H. truncatum* larvae contained detectable CCHF virus (mean virus titer $10^{1.6}$ plaque-forming units [PFU] tick). The virus was transmitted transstadially from infected larvae and was detected in 15 (1.2%) of 1,253 nymphs and 12 (0.1%) of 2,049 adults. Virus was recovered from 18 (1.9%) of 931 *H. impeltatum* nymphs, which originated from larvae that cofed with infected adults. After *H. impeltatum* nymphs cofed with infected adults, CCHF virus was detected in 21 (4.3%) of 449 (mean virus titer $10^{2.7}$ PFU tick) fed nymphs, but none of 886 adults tested after molt. Results of this study indicate that a small proportion of either larvae or nymphs may acquire CCHF infection while cofeeding on a host without a detectable viremia.

The maintenance and transmission cycles of Crimean-Congo hemorrhagic fever (CCHF) virus (family Bunyaviridae, genus *Nairovirus*) in nature are not well understood.[1] Transovarial transmission of CCHF virus in the principal *Hyalomma* spp. tick vectors could serve as a maintenance mechanism; however, transmission rates reported in the literature may be too low to have an important impact on virus maintenance.[2] Most vertebrates infected with CCHF virus in nature are not thought to be important reservoirs for the virus because very few develop high viremias. Jones and others described a novel mode of arbovirus transmission of Thogoto virus to ticks while cofeeding with infected ticks on a guinea pig that did not have a detectable viremia.[3] If CCHF virus transmission occurred between tick vectors feeding on hosts without a detectable viremia, many vertebrates could potentially serve as reservoirs. Laboratory tick-infection studies with CCHF and Dugbe (Bunyaviridae, *Nairovirus*) viruses suggest that uninfected adult ticks can acquire virus infection while cofeeding with infected adults on guinea pigs without a detectable viremia; however, possible venereal transmission or transmission by hyperparasitism could not be ruled out in these studies.[4]

Previous studies showed that CCHF virus–inoculated guinea pigs become infected but do not develop a detectable viremia (Kenyon R. C. S. Army Medical Materiel Development Activity, Fort Detrick, Frederick, MD, unpublished data). This study was designed to determine if CCHF virus could be transmitted from infected *Hyalomma* adults to immature ticks while cofeeding on a guinea pig.

MATERIALS AND METHODS

Ticks and guinea pigs

Ticks used in this study were obtained from laboratory colonies of *Hyalomma truncatum* (F16, established with 13th generation colony specimens obtained from the National Institute of Virology, Sandringham, Republic of South Africa) and *H. impeltatum* (F3, established with field collected specimens from Yonofere, Senegal) and were maintained in our laboratory. To ensure that the tick colonies were not infected with CCHF virus, adults used to establish the colony were tested for the presence of virus in cell culture and were found to be free of virus. During nonparasitic phases, the ticks were held in a plexiglass chamber maintained at a relative humidity of 92–98% and at 26°C, with a 12:12 hr L:D photoperiod. Adult guinea pigs (strain 13, 600 g or larger) were used as host animals for tick infestation in all experiments.

Virus

The CCHF virus (strain IbAr 10200) used in this study was isolated from *H. excavatum* (as *H. anatolicum excavatum*) near Sokoto, Nigeria.[3] The reference stock was obtained from the Yale Arbovirus Research Unit (New Haven, CT) as lyophilized third mouse brain passage material. The virus was further passed 15 times in newborn mice by the intracranial route. A working stock virus consisted of a clarified 10% suspension of triturated infected suckling mouse brain in medium 199, prepared in Hanks' balanced salt solution (H-199) with 1 mg ml of $NaHCO_3$ plus 5% heat-inactivated (56°C for 30 min) fetal bovine serum (FBS), 100 units ml of penicillin, 100 μg ml of streptomycin, and 2.5 μg ml of fungizone. The virus titer was $10^{7.3}$ plaque-forming units (PFU) ml as measured by plaque assay on SW-13 (human small cell carcinoma of the adrenal cortex) cell monolayers.[4]

Virus assay procedures

Ticks were triturated in H-199 with 10% FBS plus 100 units ml of penicillin, 100 μg ml of streptomycin, 2.5 μg ml of fungizone, and 50 μg ml of gentamicin (PSFG) (2 ml for adults and 1 ml for immature stages) in tissue grinders, and suspensions were prepared according to the methods described by Logan and others.[5] Aliquots of 0.1 ml of the suspensions were inoculated onto SW-13 monolayers in 12-well plates, adsorbed for 1 hr at 36°C, then overlaid with 0.6% Seakem (FMC Bioproducts, Rockland, ME) agarose in Eagle's minimum essential medium with nonessential amino acids, 10% FBS, 0.1% L-glutamine, and PSFG. Cell cultures were incubated at 36°C in a humidified CO_2 incubator for plaque development and were stained after four days with another overlay of the above medium containing 330 μg ml of neutral red. Plaques were counted after an additional day of incubation. Virus recovered from tick suspensions was identified in a plaque-reduction neutralization test with anti-CCHF virus mouse hyperimmune ascitic fluid. Serum samples were obtained from host guinea pigs one 1 day before infestation and at seven, 14, 21, and 28 days after tick feeding, and were tested for antibody to CCHF virus by indirect immunofluorescence[11] and antibody-capture enzyme-linked immunosorbent assay (ELISA).[12]

Infection and transmission experiments

Adult *H. truncatum* and *H. impeltatum* ticks were infected with CCHF virus by intracoelomic inoculation through the coxal membrane of the hind leg with a fine-tipped syringe pulled from 0.6-mm diameter capillary tubes. Each tick received approximately 0.27 μl of stock virus suspension. Samples of inoculated ticks were assayed immediately after inoculation and at seven, 14, and 21 days to determine viral titers.

Three experiments were conducted seven days after inoculation of adult ticks with CCHF virus because previous studies found that viral titers in ticks attained maximum levels at this time.[3] In experiment 1, *H. truncatum* larvae (approximately 1,000 guinea pig) were cofed, on each of six guinea pigs, with three pairs of virus-inoculated *H. truncatum* adults. In experiment 2, *H. impeltatum* larvae (approximately 1,000 guinea pig) were cofed, on each of two guinea pigs, with three pairs of virus-inoculated *H. impeltatum* adults. In experiment 3, *H. impeltatum* nymphs (approximately 300 guinea pig) were cofed, on each of six guinea pigs, with five pairs of virus-inoculated *H. impeltatum* adults. In each experiment, immature forms were fed on guinea pigs two days after infected adults had attached to the host.

Previous studies have shown that the mean feeding times for *H. truncatum* larvae and adults are four and eight days, respectively.[7] The mean feeding times for *H. impeltatum* larvae, nymphs and adults are six, seven, and eight days, respectively.[7] Most immature forms dropped off the host from one day before to approximately one day after infected adults dropped off. As replete larvae or nymphs dropped off, they were collected and sampled daily for virus isolation until drop off was complete. The number of immature forms that were assayed for virus as they dropped off the host after cofeeding with infected adults varied; however, approximately 5% of the larvae and 30% of the nymphs that dropped off on a given day were assayed. After molting, the ticks were placed for attachment on guinea pigs not previously infected with CCHF virus or not previously infested with ticks, and the process repeated to the adult stage.

This research was conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to animals and experiments involving animals and adheres to

TABLE 1

Infection rates in Hyalomma truncatum *ticks after exposure as larvae to Crimean-Congo hemorrhagic virus while cofeeding with infected adults**

Life stage	No. infected no. tested (%)	Mean log PFU/tick	Transmission to host†
Fed larvae	3/370 (0.8)	1.6	6/6
Unfed nymph (152 pools)	2/781 (0.3)	2.3	–
Unfed nymph individuals	(0)	< 0.7	–
Fed nymph	14/1,109 (1.3)	1.8	2/4
Unfed adult	7/1,756 (0.4)	2.1	–
Fed adult	5/293 (1.7)	1.9	1/6

* PFU = plaque-forming units; – = could not be evaluated in unfed nymphs and adults.
† Inoculated adults transmitted the virus while cofeeding with uninfected larvae.

principles stated in the *Guide for the Care and Use of Laboratory Animals*, NIH Publication 86-23, 1985 edition.

RESULTS

The mean virus titers in CCHF-inoculated adult *H. truncatum* and *H. impeltatum* were $10^{1.9}$ and $10^{1.6}$ PFU/tick, respectively, when assayed at the time of guinea pig infestation. In each of the three experiments, inoculated adults transmitted CCHF virus to guinea pigs, as evidenced by detection of antibodies to the virus 21 days after tick infestation (Tables 1–3). No virus was detected in the guinea pig sera when tested by plaque assay during the period of tick feeding.

Hyalomma truncatum larvae (3 [0.8%] of 370) became infected while simultaneously feeding on guinea pigs with previously infected *H. truncatum* adults (Table 1). Virus was transstadially transmitted subsequently to nymphs and adults. Nymphal ticks transmitted virus to 50% (2 of 4) and adult ticks transmitted virus to 17% (1 of 6), of the guinea pigs used as hosts.

No virus was detected in 134 replete *H. impeltatum* larvae that cofed with inoculated adults; however, virus was recovered from nymphs from molted larvae (Table 2). After a bloodmeal on naive guinea pigs, 15 of 850 replete nymphal ticks had detectable CCHF virus infections and 1 of 4 of the host animals seroconverted, indicating that virus transmission had occurred. No virus was detected in adults exposed as larvae; however, 75% (3 of 4) of the guinea pigs used as hosts contained antibody to CCHF virus after adult tick feeding.

TABLE 2

Infection rates in Hyalomma impeltatum *ticks after exposure as larvae to Crimean-Congo hemorrhagic fever virus while cofeeding with infected adults**

Life stage	No. infected no. tested (%)	Mean log PFU/tick	Transmission to host†
Fed larvae	0/134 (0)	< 0.7	2/2
Unfed nymph (96 pools)	3/503 (0.6)	1.0	–
Fed nymph	15/850 (1.8)	2.8	1/4
Unfed adult	0/365 (0)	< 0.7	–
Fed adult	0/128 (0)	< 0.7	3/4

* PFU = plaque-forming units; – = could not be evaluated in unfed nymphs and adults.
† Inoculated adults transmitted the virus while cofeeding with uninfected larvae.

In experiment 3, more than 4% (21 of 489) of the *H. impeltatum* nymphs that cofed with inoculated adults contained detectable virus (Table 3). Although no virus was found in the 886 adults derived from exposed nymphs, CCHF virus was transmitted to 40% (2 of 5) of the guinea pigs used as hosts.

DISCUSSION

In this study, *H. truncatum* larvae and *H. impeltatum* larvae and nymphs became infected with CCHF virus while cofeeding on guinea pigs with adult ticks inoculated seven days previously with CCHF virus. No viremia was detectable using SW-13 cell culture; however, guinea pig serum inoculated into adult *H. truncatum* ticks, which were then incubated for seven days, triturated, and inoculated onto SW-13 cell cultures, yielded virus. As demonstrated by the detection of antibodies to CCHF virus, inoculated adult ticks transmitted virus to guinea pigs. These observations are similar to the findings reported by Jones and others,[3] in which Thogoto virus in-

TABLE 3

Infection rates in Hyalomma impeltatum *ticks after exposure as nymphs to Crimean-Congo hemorrhagic fever virus while cofeeding with infected adults**

Life stage	No. infected no. tested (%)	Mean log PFU/tick	Transmission to host†
Fed nymph	21/489 (4.3)	1.7	6/6
Unfed adult	0/754 (0)	< 0.7	–
Fed adult	0/132 (0)	< 0.7	2/5

* PFU = plaque-forming units; – = could not be evaluated in unfed nymphs and adults.
† Inoculated adults transmitted the virus while cofeeding with uninfected larvae.

fection in guinea pigs also produced very low-level virus replication.

Our inability to detect CCHF virus in unfed nymphs in experiment 1 and adults in experiments 2 and 3 may result from low viral titers in ticks and low sensitivity of our viral assay on SW-13 cells. Serologic evidence of virus transmission to guinea pigs demonstrated that these stages were infected. The previous studies that assayed ticks infected with CCHF virus in SW-13 cells found that some low-titered virus infections were not detected.[5,10]

Detecting CCHF virus infection in nymphs and adults arising from ticks exposed to CCHF virus as either larvae or nymphs demonstrates that the ticks became infected and that the virus was transmitted transstadially. Because virus recovery rates in unfed nymphs and adults were lower than rates in fed nymphs and adults, it is possible that transmission may also have occurred between infected and uninfected, cofeeding nymphs (experiments 1,2) and cofeeding adults (experiment 1). Jones and others found that salivary gland enhancement of Thogoto virus transmission in a nonviremic host occurred after ticks were attached to a host for 4–6 days.[13] *Hyalomma truncatum* nymphs and adult females feed an average of eight days[5] and *H. impeltatum* nymphs feed for seven days.[7] Virus titers in nymphs and adults after molting from larvae and nymphs were slightly higher than those observed in larvae and nymphs exposed to virus. Similarly, the demonstration that both nymphs and adults, exposed as larvae or nymphs, transmitted CCHF virus to guinea pigs indicates that the virus infection in ticks was disseminated to the salivary glands.

The use of cofeeding adults and immature forms excluded the possibility that venereal transmission was the mechanism by which uninfected ticks became infected. *Hyperparasitism* still could have occurred, since adults and immature forms were placed on the guinea pig within the same feeding cell; however, immature forms were closely monitored after infestation and those that failed to attach were removed after 24 hr. Previous studies have reported that CCHF virus replication in *H. truncatum* and *H. impeltatum* increases during blood feeding.[5,10] Increased viral replication in ticks during blood feeding may have enhanced the transmission of the virus to uninfected ticks.

Although *Hyalomma* ticks can be infected with CCHF virus while feeding on newborn mice inoculated with the virus, we have not been able to infect ticks on adult laboratory animals inoculated with the virus.[10] The demonstration of virus transmission to uninfected ticks through cofeeding on a host without a detectable viremia marks our first success in infecting ticks on adult animals. These results provide supporting laboratory evidence for the epidemiologic observations incriminating *H. truncatum* and *H. impeltatum* as vectors of CCHF virus. The overall infection rate in ticks exposed as larvae to a nonviremic host was 0.7% in *H. truncatum* and 0.9% in *H. impeltatum*.

The observation that transmission of CCHF virus occurred in two different tick species during cofeeding on a host with an undetectable level of virus when assayed in SW-13 cells suggests that this mode of transmission might be more than an isolated phenomenon. The transmission reported here may be an important observation in the ecology of CCHF virus because the distribution and population densities of *Hyalomma* spp. ticks are closely associated with the occurrence of CCHF virus, particularly in Africa.[1] *Hyalomma* spp., including *H. truncatum* and *H. impeltatum*, are found on a wide variety of hosts[7] and are considered important vectors of CCHF, based upon experimental studies[5,7] and field isolations of the virus.[1,17]

Although antibodies to CCHF virus are found in numerous species of vertebrates, very few are thought to develop CCHF viremias high enough to be involved in the transmission of the virus.[5,7,14] Similar results were observed by Gonzalez and others,[4] who found that uninfected female *H. truncatum* became infected while cofeeding with infected males on rabbits. Our results suggest that a vertebrate could be involved in CCHF virus transmission to ticks, even though it may not develop a high viremia. The transmission we observed in ticks cofeeding on a host without a detectable viremia suggests that many more vertebrates than previously considered could serve as amplifying hosts of the virus, enhancing the survival of virus in nature.

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